INS A1

PROTEIN

Field of the invention

The invention relates to a new family of proteins which are able to bind to α_2-macroglobulin and peptide fragments of this family of proteins. The invention also relates to the use of a protein or peptide derived from an α_2-macroglobulin binding protein for use in a vaccine composition for group A streptococcus.

Background of the invention

Streptococcus pyogenes (group A Streptococcus) (GAS) is an important human pathogen which causes a variety of diseases such as pharyngitis, impetigo, scarlatina and erysipelas. More severe infections caused by this organism are necrotizing fasciitis and streptococcal toxic shock like syndrome.

S. pyogenes binds several human plasma proteins via its surface proteins. *S. pyogenes* binds to α_2 macroglobulin (α_2M) which is a proteinase inhibitor. α_2M is a glycoprotein of 718 kD composed of two pairs of identical subunits held together by disulphide bonds.

Previous studies have identified a non-proteolytic cell wall protein of 78 kD of Group A Streptococci which binds to α_2M:Chhatwal *et al* J. Bacteriol. (1987) 169(8) 3691-5.

Summary of the invention

The present inventors have identified a new group of proteins which are expressed on the surface of some strains of Group A streptococcus, *S.pyogenes*. These proteins have the ability to bind to α_2-macroglobulin, and show some homology to protein G of Group G streptococcus. The new protein derived from *S.pyogenes* has been termed protein GRAB by the present inventors. The gene encoding this protein is referred to as *grab*.

The present invention relates in particular to a protein which is capable of binding α_2M and which comprises the amino acid sequence of SEQ ID No. 1 or a functional variant thereof. In preferred embodiments, the protein comprises the amino acid sequence of SEQ ID No. 2 or a functional variant thereof, and/or one or

more tandem repeats having the amino acid sequence of SEQ ID No 3 or a variant thereof. The protein of the invention may further comprise a cell membrane anchor region and a hydrophobic transmembrane region. Preferably, the protein consists of the amino acid sequence of any of SEQ ID Nos. 1 to 11 and variants thereof.

The invention also provides:

- a peptide comprising a fragment of at least 6 amino acids in length of a protein having the amino acid sequence of (a) any of SEQ ID Nos 1 to 11 or (b) a variant of any of SEQ ID Nos 1 to 11;
- a peptide as defined above having the ability to generate an immune response in an individual and vaccine compositions comprising such a peptide and methods of immunization comprising administering such a peptide to an individuals;
- a DNA sequence which codes for a protein or peptide according to the invention, said DNA sequence being selected from:
  - (a) the DNA sequence of any of SEQ ID Nos 12 to 16 or the complementary strands thereof;
  - (b) DNA sequences which selectively hybridize the DNA sequences defined in (a) or fragments thereof; and
  - (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) or (b) and which sequences code for a protein or peptide having the same amino acid sequence;
- an expression vector comprising a DNA sequence of the invention operably linked to a regulatory sequence;
- a host cell transformed with a DNA sequence of the invention or an expression vector of the invention;
- a process for producing a protein or peptide of the invention, comprising culturing a host cell of the invention under conditions to provide for expression of the desired protein or peptide.
- an antibody capable of binding a peptide or protein of the invention and a method of treating an individual by immunotherapy using the

antibody.

Description of the figures

Fig. 1 . The binding of radiolabeled α_2M to 10^9 bacteria of different strains of *S. pyogenes* grown to early stationary phase is presented in A (bars represent +SEM, n=3). In B the binding of radiolabeled α_2M to $2x10^8$ KTL3 bacteria was competed with α_2M and with protein G (+/- SD, n=3). In C the scatchard plot for the reaction between α_2M and 10^9 KTL3 bacteria is shown. The shape of the plot suggests two binding sites with different affinities (K_a=$2.0x10^8M^{-1}$ and $5.3x10^6M^{-1}$ respectively).

Fig. 2. A schematic comparison between protein GRAB and protein G is shown in A. The complete nucleotide and amino acid sequence of *grab*/protein GRAB is shown in B.

Fig. 3. Different strains of S. pyogenes were subjected to PCR and the results are set out in (A). From all strains, except from the AP9 strain, a product of between 500 and 850 bp in size could be amplified (A). Schematic comparison of the mature protein GRAB (amino acids 34-188 in Fig 2B) encoded by these strains is shown in B.

Fig. 4. MBP-GRAB was used to inhibit the binding of radiolabeled α_2M to $2x10^8$ KTL3 bacteria. Similarly one synthetic peptide (aa 34-56 in Fig 2B) was able to compete for the binding of α_2M although less efficiency that MBP-GRAB. while an overlapping peptide (aa 51-68 in Fig 2B) did not compete for the binding. Bars represent +/- SD, n=3.

Fig. 5. An internal fragment of *grab*, lacking the part of the gene coding for the cell wall attachment, was cloned into the streptococcal suicide plasmid pFW13 to generate FW-*grab*. pFW-grab was transformed into KTL3 bacteria, to generate MR4. MR4 was completely devoid of α_2M binding as shown (+SD, n=3).

Fig. 6. The binding of the radiolabeled fibrinogen was measured after trypsin treatment of KTL3 or MR4 bacteria. Some bacteria were preincubated with α_2M (+α_2M) and some were not. As can be seen, preincubation of KTL3 with α_2M protected the M protein, and thus fibrinogen binding, from trypsin degradation. α_2M pretreatment of MR4 did not affect the fibrinogen binding (+SD n=3).

Fig. 7. Radiolabeled and activated SCP was added to KTL3 (1), MR4 (3), or the same bacteria preincubated with α_2M (2 and 4 respectively). The binding of SCP was significantly higher to KTL3 bacteria that had been preincubated with α_2M (+SD, n=3).

Fig. 8. Shows the results of an assay of sheep anti-DSP 18. peptide sera on a GRAB coated plate.

Fig. 9. Shows the results of ELISA using

Fig. 10. Shows the serum antibody response in mice immunised with a protein or peptide of the invention.

Fig. 11. Shows the results of opsonization of log phase group A streptococcus by sera to a protein or peptide of the invention.

Detailed description of the invention.

The invention relates generally to proteins which bind α_2M. Binding of α_2M to bacteria or proteins can be determined using radiolabeled α_2M. For example, bacteria can be incubated with radiolabeled α_2M. After centrifugation, radioactivity of the pellets can be determined and expressed as a percentage of added activity over control samples containing no bacteria. The binding of radiolabeled α_2M could also be competed with non-labeled α_2M or other protein such as protein G. This suggests that the novel protein binds to the same site as does protein G or an overlapping site on α_2M. It suggests that the α_2M binding of Group A streptococcus (GAS) bacteria is attributable to a protein G-like protein. This is confirmed by the examples below, which suggest that protein GRAB is the only α_2M binding protein of GAS.

The Examples below also describe the generation of a mutant strain of Group A Streptococcus, *S. pyogenes* which no longer expresses protein GRAB on its surface. This could also be used as a control. Binding of α_2M to proteins can be assessed by immobilizing the proteins on a support such as nitrocellulose and probing with radiolabeled α_2M. After washing, the radioactivity of the bound protein can be determined to give an indication of specific binding of α_2M to bound protein. The Examples below describe one method for evaluation of the binding of α_2M to both bacteria or proteins.

The inventors have identified a region of protein GRAB which can inhibit α_2M binding to *S.pyogenes* which express protein GRAB. The sequence for this region is set out in SEQ ID No.1. The invention relates to proteins comprising the amino acid sequence of SEQ ID No.1 and variants of this sequence. The term variants is used to cover related amino acid sequences which may differ from the exact sequence of SEQ ID No. 1. Variants according to the invention can be identified in a number of different ways as explained in more detail below.

In another aspect of the invention, a protein or peptide is provided to generate an immune response, preferably a protective immune response to group A streptococcus in an individual. Preferably, the group A streptococcus (*S. Pyogenes*) is one which expresses protein GRAB as defined herein. A protein or peptide for use in a vaccine formulation is one which is capable of generating an immune response in an individual. Suitable proteins or peptides are derived from protein GRAB or variants thereof. Such proteins or peptides for use in a vaccine formulation may or may not retain the ability to bind α_2M. A protein or peptide of the invention may also be used to generate an antibody to protein GRAB which may be used in the diagnosis or treatment by immunotherapy of GAS infection.

Variant sequences may be identified in protein GRAB produced from other strains of *S. pyogenes*. Partial sequence data for protein GRAB isolated from a number of different strains of *S.pyogenes* is set out in SEQ ID Nos. 7-11. Each of these sequences includes the sequence of SEQ ID No.1 except for a single residue difference in protein GRAB derived from AP1 (SEQ ID No 9). The variation from SEQ ID No.1 is the replacement of isoleucine for threonine at position 18. This sequence is one example of a variant sequence of the invention.

The Examples below show expression of protein GRAB from a number of other strains of *S.pyogenes*. Protein GRAB from these strains may also be used to identify an α_2M binding region or a region which inhibits α_2M binding to protein GRAB expressing *S.pyogenes*, and also to identify sequences which are variants of SEQ ID No.1. The relevant region from such protein GRABs can be identified by alignment of the amino acid sequence data obtained for protein GRAB from other strains with the sequences set out in SEQ ID Nos 1-11. When the maximum

alignment is achieved, the relevant region of the protein comprising a variant on SEQ ID No. 1 can readily be identified.

In an alternative aspect of the invention, proteins and variant sequences are those which can be used in a vaccine formulation and against which an immune response, preferably a protective immune response to group A streptococcus is generated on administration of the peptide to an individual. In this aspect of the invention, the protein or peptide may no longer retain the ability to bind α_2M. Such sequences may be derived as described below to identify sequences which do bind α_2M but may be modified such that the ability to bind α_2M is lost through deletion, substitution or insertion in the amino acid sequence of a protein which does maintain the ability to bind α_2M. Particularly preferred are fragments of protein GRAB which are described in more detail below.

Protein GRAB from other *S.pyogenes* strains can be identified, firstly by investigating the α_2M binding properties of the strain. Subsequently the desired sequence information can be obtained by cloning the genomic DNA and conducting PCR using primers which hybridize to sections of DNA encoding the peptides set out in SEQ ID Nos 1-11. The Examples below demonstrate identification and partial sequencing of protein GRAB derived from a number of *S.pyogenes* strains. In particular, primers hybridizing to the sequences set out in SEQ ID Nos. 17-21 can be used in the cloning and sequencing of protein GRAB from other *S.pyogenes* strains. The region of protein GRAB identified in SEQ ID No. 1 is highly conserved between the different strains of *S.pyogenes*. In general the variant sequences derived from other *S.pyogenes* would be expected to differ by 1, 2, 3, 4, or up to 5 amino acids from SEQ ID No 1, and more likely by 1 or 2 amino acid residues. Proteins having this variant sequence retain the ability to bind to α_2M.

Variants of SEQ ID No.1 also include sequences which vary from SEQ ID No.1 but which are not necessarily derived from naturally occurring protein GRAB. These variants may be described as having a % homology to SEQ ID No.1 or having a number of substitutions within this sequence. Alternatively a variant may be encoded by a polynucleotides which hybridizes to any one of SEQ ID No 12-16, which is discussed in more detail below.

A variant of SEQ ID No. 1 is one which has at least 78 % homology thereto. Preferably the variant will be at least 83 or 87% and more preferably 91 or 96% homologous thereto. Methods of measuring protein homology are well known in the art and it will be well understood by those of skill in the art that in the present context, homology is calculated on the basis of amino acid identity ("hard homology").

Amino acid substitutions may be made, for example from 1, 2 or 3 up to 4, 5 or 6 substitutions in SEQ ID No.1. The modified sequence generally retains the ability to bind α_2M. Conservative substitutions may be made, for example according to the following Table:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

Preferably, the proteins of the invention comprise an extension to SEQ ID No.1. Thus the protein preferably comprises SEQ ID No.2. The protein may also comprise sequences which are fragments of SEQ ID No.2 which incorporate at least all of SEQ ID No 1. The protein may therefore comprise a sequence of 25 amino acids commencing at the N-terminal of SEQ ID No.2 and may comprise 30, 35, 40, 45 or 50 amino acids of SEQ ID No. 2 up to the entire sequence of 58 amino acids of SEQ ID No 2. The proteins of the invention may also comprise variants of such sequences.

The variants can be defined in a similar manner to the variants if SEQ ID No.

1. Thus the variants may comprise variant sequences derived from other strains of *S.pyogenes*. For example the Examples describe protein GRAB derived from a number of different strains of *S.pyogenes*. SEQ ID Nos. 7-11 set out sequence data for some of these strains. Alignment with SEQ ID No.2 to give the maximum identity in alignment will allow those of skill in the art to determine variant sequences of SEQ ID No. 2.

Other variants can be identified as outlined above from other *S.pyogenes* strains by looking for α_2M binding and cloning and sequencing as before. α_2M binding of variant proteins can be determined by expression cloning and western blotting of the recombinant protein using radiolabeled α_2M.

Variants can also be identified by % homology or have substitutions as described above. A greater number of substitutions or lower % homology can be tolerated for longer sequences such as larger fragments of SEQ ID No. 2 or the entire sequence. For example, 1, 2, 3 up to about 10 to 15 substitutions in SEQ ID No.2 may be incorporated. Alternatively a variant may have at least 74%, 78% or 81% homology, and preferably has at least 85% or 90%, 95%, 97% or 98% homology. As before the variants preferably maintain the ability to bind α_2M.

The proteins of the invention may also comprise the sequence of SEQ ID No 3 or a variant sequence thereof, or a fragment of either sequence. Preferably the proteins of the present invention further comprise two or more tandem repeats of the sequence SEQ ID No. 3 and variants thereof. The proteins isolated from *S.pyogenes* and termed protein GRAB have at least two repeated sequences adjacent to the C-terminus of SEQ ID No.2 or variant thereof. These repeat sequences have the sequence set out in SEQ ID No.3 or a variant thereof. As can be seen from SEQ ID Nos 7-11, the sequence can show some variation within each repeat both in a single protein GRAB and also between protein GRAB isolated from different strains of *S.pyogenes*. Thus the term repeat as used herein does not mean that an exact repeat of the same sequence is present but simply that a sequence and one or more variants thereof are present, preferably in tandem.

The protein may comprise 2, 3, 4, 5 or 6 or more repeat sequences. Each repeat sequence is generally 28 amino acids in length but may be from 21 up to 35

amino acids in length. Within each protein the length of the repeat sequence therein may vary. For example a protein may comprise a sequence of 28 amino acids followed by a variant repeat sequence of 35 amino acids. The repeat sequence of the invention may adapt a coiled coil structure. This structure is based on heptadic structure of amino acid units which allow the protein to form a coil.

Variants of the repeat sequence of SEQ ID No 3 derived from other strains of *S.pyogenes* can be readily identified by reference to the sequences set out in SEQ ID Nos. 7-11. Each of these sequences has at least two repeats. Repeat sequences derived from protein GRAB from other *S.pyogenes* strains can be identified as outlined above through cloning and sequencing. Other variants encompassed by the present invention are sequences identified by % homology or substitutions as outlined above for SEQ ID No.1 or Seq ID No. 2. For example a variant may be a repeat having at least 60% homology, preferably at least 70 or 75% up to 85 or 90% up to at least 96% homology with SEQ ID No 3. A variant may have 1, 2 or 3 up to 6, 7, 8 or 9 substitutions in SEQ ID No 3. Preferably the variant retains the heptad structure allowing the region to form a coiled structure. A sequence encoded by a polynucleotide which hybridizes with a polynucleotide encoding a repeat sequence as described herein is also a variant of the invention.

The proteins of the invention may also comprise additional regions such as a cell membrane anchor region and a transmembrane region. The sequence of SEQ ID No.4 comprises a protein having an α_1M binding region, a repeat sequence region and a cell membrane anchor region and transmembrane region. The proteins of the invention can comprise variants of the cell membrane anchor and transmembrane regions as defined above for the other sequences of the protein. Such variants preferably retain the cell membrane anchor function and/or transmembrane function.

It may be desirable to ensure that the transmembrane regions or anchor regions are not present in the protein. For example, if a protein is desired which has the ability to bind α_1M but which will be excreted from the bacterial cell in which it is expressed, the anchor and transmembrane regions are preferably not expressed as part of the protein.

In one preferred embodiment of the present invention, the protein consists

essentially of any one of SEQ ID Nos 1-11 and variants thereof as defined above.

The present invention also relates to peptides comprising a fragment of at least 6 amino acids in length of a protein of the invention. In particular, the invention relates to such a peptide comprising a fragment of the protein having the sequence of any one of SEQ ID Nos. 1-11 and variants thereof. Preferably, the fragment will be at least 10, for example at least 12 or 15, amino acids in length. The fragment may be up to 20, 30, 40, 60 or 150 amino acids in length.

In a preferred embodiment, a peptide of the invention has the ability to bind α_2M. This binding can be determined as outlined above. As will be readily appreciated by one skilled in the art, peptides of shorter length preferably comprise a fragment of protein GRAB derived from *S.pyogenes*. For longer peptides, the sequences may show greater variation as set out above, such as a smaller % homology or greater number of substitutions.

In an alternative aspect of the invention, a peptide has the ability to generate an immune response on administration to an individual and preferably to generate a protective immune response in an individual. Such a peptide may additionally retain the ability to bind α_2M. However, such binding is not necessarily required. A peptide for use in this embodiment comprises a fragment of the protein having the sequence of any one of SEQ ID Nos. 1-11 and variants thereof as described above. Such a fragment is at least 6 amino acids in length and preferably the fragment will be at least 10, for example at least 12 or 15 up to 20, 30 or 40 amino acids in length. Longer fragments such as fragments up to 60 or 150 amino acids in length may also be used. A variant of the sequences of the SEQ ID Nos. 1-11 are described above with reference to the ability to bind α_2M. However such variants for use in a vaccine composition do not need to retain the ability to bind α_2M. Such a variant sequence for use in a vaccine is one which has the ability to generate an immune response on administration to an individual.

Preferably, a peptide for incorporation into a vaccine formulation is one which is derived from the extra cellular region of protein GRAB. Preferred peptides include DSP18, SEQ ID No. 22; EKL 24, SEQ ID No. 23; EKL18, SEQ ID No 24; EER17, SEQ ID No 25 and KKT18, SEQ ID No. 26. Preferred peptides also include

variants of these peptides and fragments of the proteins of the invention which incorporate part or all of SEQ ID 22 to 26. In a particular preferred embodiment, the invention relates to a peptide which is derived from the region of protein GRAB located C-terminal and adjacent to the α_2M binding region. Such a peptide is exemplified by the peptide of SEQ ID No. 23, 24 or 25. In one aspect of the invention, a peptide for use in a vaccine composition does not retain the ability to bind α_2M. Binding to α_2M site may reduce the effectiveness of the peptide if there is a large amount of free α_2M which may simply bind to such administered peptide and reduce its efficacy as a vaccine composition, or bind to GRAB in vivo obscuring the target epitope.

Peptides for use in a vaccine composition in accordance with the invention may comprise longer peptide sequences derived from protein GRAB or may encompass the full length protein. Preferably however the vaccine composition comprises a fragment of protein GRAB as defined above. A peptide for use in generating an immune response may be identified by immunisation studies. For example a candidate peptide may be administered to an animal and subsequently the antibody or T-cell response generated which is specific for the peptide may be determined. Antiserum generated following administration of a peptide to an animal may be evaluated for the ability to bind the peptide or to bind protein GRAB. Subsequently the animal may be challenged with Group A streptococcus to evaluate whether a protective immune response has been generated.

In another embodiment, the peptide comprises a fragment of the repeat sequence or variant thereof, as described above. In this embodiment the peptide may comprise an entire repeat sequence that is of about 28 amino acids in length as outlined above, or two or more repeat sequences in tandem.

Proteins and polypeptides of the invention may be in substantially isolated form. It will be well understood that the proteins or peptides may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein or peptide and still be regarded as substantially isolated. A protein or peptide of the invention may also be in substantially purified form, in which case it will generally comprise the protein or peptide in a preparation in which more than 90%, for

example more than 95%, 98% or 99%, by weight of the protein or peptide in the preparation is a protein or peptide of the invention.

Proteins or peptides of the invention may be modified for example by the addition of one or more histidine residues to assist in their identification or purification or by the addition of a signal sequence to promote their secretion from a cell. Some of the signal sequences derived from protein GRAB from a number of *S.pyogenes* strains are set out in SEQ ID Nos. 7-11, and can be seen located N-terminally from the α_2M binding region or SEQ ID No.1 or variant thereof. It may be desirable to provide the peptides or proteins in a form suitable for attachment to a solid support. The proteins or peptides may thus be modified to enhance their binding to a solid support for example by the addition of a cystine residue.

A protein or peptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the protein or peptide to be detected. Suitable labels include radioisotopes such as ^{125}I,^{35}S or enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled proteins and peptides of the invention may be used in assays for example to assess levels of α_2M. In such assays it may be preferred to provide the peptides attached to a solid support. The present invention also relates to such labelled and/or immobilized protein and peptides packaged in the form of a kit in a container. The kit may optionally contain other suitable reagent(s). control(s) or instructions and the like.

The proteins of the present invention may be isolated from *S.pyogenes* expressing the protein. Proteins and peptides of the invention may be prepared as fragments of such isolated proteins. The proteins and peptides of the invention may also be made synthetically or by recombinant means. The amino acid sequence of proteins and polypeptides of the invention may be modified to include non-naturally occurring amino acids or to increase the stability of the compound. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The proteins or peptides of the invention may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the

C to N orientation. This is conventional in the art for producing such proteins or peptides.

A number of side chain modifications are known in the art and may be made to the side chains of the proteins or peptides of the present invention. Such modifications include, for example, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride.

The invention also relates to polynucleotides encoding the proteins and peptides of the invention and their use in producing the proteins and peptides of the invention by recombinant means. In particular the invention relates to (a) the DNA sequence of any one of SEQ ID Nos 12 to 16 or the complementary strands thereof: (b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) or (b) and which sequences code for a polypeptide having the same amino acid sequence. Hybridization is typically carried out under conditions of high stringency, such as hybridization buffer of 6x SSC, 0.5% SDS at 65°C. Hybridization conditions equivalent to the conditions described herein could also be used to identify the polynucleotides of the invention.

Polynucleotides of the invention may also comprise corresponding RNA to these DNA sequences. The polynucleotides may be single or double stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

Preferred polynucleotides of the invention include polynucleotides encoding any of the proteins and peptides described above. Those skilled in the art will understand that numerous different polynucleotides can encode the same protein or peptide as a result of degeneracy of the genetic code.

A nucleotide sequence capable of selectively hybridizing to the DNA sequence of any one of SEQ ID Nos: 12 to 16 or to a DNA sequence complementary to any one of those sequences will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% or 97%, homologous to such a DNA sequence. This homology may typically be over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous nucleotides of the said DNA sequence.

Any combination of the above mentioned degrees of homology and minimum sized may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher homology over longer lengths) being preferred. Thus for example a polynucleotide which is at least 80% homologous over 25, preferably over 30 nucleotides forms one aspect of the invention, as does a polynucleotide which is at least 90% homologous over 40 nucleotides.

Homologues of polynucleotide or protein sequences are referred to herein. Such homologues typically have at least 70% homology, preferably at least 80, 90%, 95%, 97% or 99% homology, for example over a region of at least 15, 20, 30, 100 more contiguous nucleotides or amino acids. The homology may calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings).

(Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (such as identifying equivalent or corresponding sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold

(Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSP's containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm paramters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an idication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein. Examples of primers of the invention are set out in SEQ ID Nos 17 to 21.

Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will

involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the *grab* which it is desired to clone, bringing the primers into contact with DNA obtained from a bacterial cell, preferably of an *S.pyogenes* strain, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al*, 1989.

Polynucleotides or primers of the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using techniques known *per se*.

Polynucleotides or primers of the invention or fragments thereof labelled or unlabelled may be used by a person skilled in the art in nucleic acid-based tests for detecting or sequencing *grab* in a bacterial sample.

Such tests for detecting generally comprise bringing a bacterial sample containing DNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridizing conditions an detecting any duplex formed between the probe and nucleic acid in the sample. Such detection may be achieved using techniques such as PCR or by immobilizing the probe on a solid support, removing nucleic acid in the sample which is not hybridized to the probe, and then detecting nucleic acid which was hybridized to the probe. Alternatively, the sample nucleic acid may be immobilized on a solid support, and the amount of probe bound to such a support can be detected.

The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may also contain suitable reagents for treating the sample to be probed, hybridizing the probe to nucleic acid in the sample, control reagents, instructions, and the like.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about the replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect cells such as Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

Such vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and optionally recovering the expressed polypeptides.

The vectors may be for example, plasmid or virus vectors provided with an origin or replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used in vitro, for example for the production of RNA or used to transfect or transform a host cell.

Promoters/enhancers and other expression regulation signals may be selected

to be compatible with the host cell for which the expression vector is designed. For example prokaryotic promoters may be used, in particular those suitable for use in *E.coli* strains. When expression of the polypeptides of the invention is carried out in mammalian cells, mammalian promoters may be used. Tissues-specific promoters, for example hepatocyte cell-specific promoters, may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

Vaccines may be prepared from one or more of the proteins or peptides of the invention and a physiologically acceptable carrier or diluent. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in a liposome. The active immunogenic ingredient may be mixed with an excipient which is pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, of the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminium hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide

containing a GRAB antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parentally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. a a suspension. Reconstitution is preferably effected in buffer.

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

The proteins or peptides of the invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salt (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

The vaccines are administered in a manner compatible with the dosage formulation and in such amount will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5μg to

100mg, preferably 250μg to10mg of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgement of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple does schedule is one in which a primary course of vaccination may be 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example at 1 to 4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgement of the practitioner.

The proteins and peptides of the invention which have the ability to bind α_2M may be used to purify α_2M from a sample. Typically, the proteins or peptides of the invention will be bound to a solid support. A sample potentially containing α_2M can be applied to the support to remove α_2M from the sample. If desired, α_2M can then be released from the support for further use.

The proteins and peptides of the invention which are capable of inhibiting binding of α_2M to the surface of streptococci may be used to inhibit such α_2M binding to the bacterial surface. The proteins and peptides can also be used in competition studies to identify other agents which may effect α_2M binding.

The proteins and peptides of the invention can be used to generate antibodies against strains of *S.pyogenes*. The poylnucleotides of the invention can be used in the production of the proteins and peptides of the invention. As outlined above, they may also be used as primers or probes for identification of related genes to *grab*.

The nucleotide sequences of the invention and expression vectors can also be used as vaccine formulations as outlined above. The vaccines may comprise naked nucleotide sequences or be in combination with cationic lipids, polymers or targeting systems. The vaccines may be delivered by any technique suitable for delivery of nucleic acid vaccines.

The immunogenic polypeptides prepared as described above can be used to

produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide of the invention. Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to the polypeptide contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Monoclonal antibodies directed against Streptococcal epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against polypeptides of the invention can be screened for various properties; i.e., for isotype and epitope affinity. Preferably the antibody is specific for a GRAB protein epitope.

Antibodies, both monoclonal and polyclonal, which are directed against polypeptides of the invention are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired.

Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful for treatment of Streptococci, as well as for an elucidation of the immunogenic regions of polypeptides of the invention.

It is also possible to use fragments of the antibodies described above, for example, Fab fragments. Antibodies generated to a peptide of the invention may be administered to an individual to treat GAS infection by passive immuno therapy. The antibodies of the invention may be formulated with a pharmaceutically acceptable carrier and delivered in the same way as set out above for the vaccine compositions. Preferably the antibody is administered in an amount effective to

ameliorate GAS infection in the individual.

Examples

The following Examples illustrate the invention.

Example 1

S. pyogenes bind native α2M via a protein G like protein - Different strains of *S.pyogenes* were tested for their ability to bind radiolabeled native α2M. *S. pyogenes* strains denoted AP are from the Institute of Hygiene and Epidemiology, Prague, Czech Republic. The KTL strains are from the Finnish Institute for health, and the SF370 strain is the ATCC 700294 strain. Bacteria were harvested in early stationary phase or after overnight culture, washed in PBS with 0.05% Tween-20 and 0.02% azide (PBSAT) and resuspended in the same buffer. Concentration of bacteria was determined by spectrophotometry and $2x10^9$ or $4x10^8$ were incubated with radiolabaled α_2M in 225 μl PBSAT for 50 minutes. For competition different amounts of unlabeled inhibitor was added to the tubes. After centrifugation, radioactivity of the pellets was determined and expressed as percentages of the added activity deducing the non-specific binding to the polypropylene tubes.

The results are shown in Fig 1A. The binding ranged from 0-76 % and differed between strains even within a given serotype. No strain bound a trypsin complexed form of α_2M (data not shown).

The KTL3 strain of the clinically important M1 serotype which bound 53% of added α_2M was chosen for further studies. The binding of radiolabeled α_2M to the KTL3 strain could be competed by both non-radioactive α_2M and by protein G from the strain G148, a group G Streptococcus (Fig. 1B). The scatchard plot for the reaction between α_2M and KTL3 bacteria (Fig. 1C) suggests that two different affinities exist, one high affinity interaction $K_a=2.0x10^8M^{-1}$ and one low affinity interaction $K_a=5.3x10^6M^{-1}$. Since the binding of α_2M to the KTL3 strain could be competed by protein G, we used the protein sequence of protein G from G148 in a tBLASTn search against the Streptococcal Genome Sequencing Project database.

A gene coding for a protein with some homology to the α_2M binding E domain of protein G, as well as to the signal sequence and cell-wall attachment of protein G, was identified. The protein was termed protein GRAB from protein G

related α_2M binding protein and consisted of 217 amino acids with a deduced molecular weight of 22.8 kDa. In 2A a schematic representation of the homology between protein GRAB and protein G is shown. In Fig 2B the nucleotide and amino acid sequences are set out. The A region includes the α_2M binding region. Two repeat regions are identified R1 and R2 and are followed by the wall spanning (W) and membrane spanning (M) regions. Protein GRAB was found to contain the consensus sequence for gram-positive surface cell wall anchored proteins (LPXTGX) followed by a stretch of 19 hydrophobic amino acids and a seven residue long hydrophilic C-terminus (Fig. 2B). The first 34 amino acids of protein GRAB showed some homology to the signal sequence (Ss) of protein G and was followed by 35 amino acids with some homology to the E domain of protein G (Fig. 2B). Spacing the regions with homology to protein G two unique repeated regions of 28 amino acids were identified.

Example 2

Distribution of expression of grab - Genomic DNA was prepared from *S. pyogenes*. PCR was performed using *Taq* polymerase (Gibco-BRL, Gaithersburg, MD) and synthetic oligonucleotides hybridizing to *grab*. Primers hybridized to the following nucleotides in figure 2B primer 1: 101-125, primer 2: 101-128, primer 3: 160-185, primer 4: 594-563 and primer 5: 627-605. Restriction enzymes and ligase were from Gibco-BRL and standard ligation, transformation, and plasmid isolation methods were used. For PCR screening and for cloning in pGEM (Promega, Madison, WI) primers 1 and 5 were used. Sequencing of the pGEM-*grab* plasmids was performed using an ABI-470 prism and Taq dyed dideoxy terminator kit (Perkin and Elmer, Norwalk, CT).

The same strains that were used in the screening for α_2M binding were subjected to PCR using primers hybridizing to *grab*. A PCR product could be generated from all strains except for the AP9 strain, but the size of the product varied between 500 base pairs (bp) and 850 bp (Fig. 3A). Sequencing of the PCR product from four strains revealed that the size polymorphism was due to a variable number of 28 amino acids repeats (Fig. 3B). Comparing the sequence from these four strains

and the one presented in the Streptococcal Genome Sequencing Project it was found that protein GRAB is highly conserved. Both the C- and – terminus was nearly 100% conserved while the repeated region showed 86% identity between strains (Fig. 3B). SEQ ID Nos 7 to 11 show partial sequence data for these strains. SEQ ID Nos 12 to 16 show corresponding nucleotide sequences.

The transcription of *grab* was investigated using Northern blotting where total RNA from the KTL3 strain which bound radiolabeled α_2M and a strain that did not (AP1) was isolated from bacteria in early logarithmic phase, late logarithmic phase, early stationary phase and late stationary phase. The RNA was electrophorized, blotted, and probed with a PCR product generated from *grab* using primers 1 and 5. Detectable amounts of a transcript of approximately 600 bp of *grab* RNA was found in KTL3 bacteria but not in AP1. The expression was highest in early logarithmic phase and dropped to undetectable amounts in the late stationary phase. The same filters were probed with a probe hybridizing with 16S which verified that the same amount of RNA had been applied to each well.

Example 3

Protein GRAB binds α_2M via the extreme N-terminus - The DNA encoding the predicted mature protein GRAB (amino acids 34-189 in Fig. 2B) from the KTL3 strain was PCR cloned into the pMal-p2 vector using the EcoR1 and Pst1 sites present in primers 3 and 5 respectively. The vector was transformed into *E.coli*. For molecular cloning purposes the DH5α strain of *Escherichia coli* was used. *E.coli* were grown in Luria Bertoni broth (10g tryptone (Difco), 10g NaCl, and 5g yeast extract (Difco)/l) supplemented with 2 g glucose/l when using the pMal-p2 vector. For growth in petri dishes 15g/l of bacto agar (Difco) was added. When E.coli contained plasmid 100 μg/ml ampicillin (Sigma, St. Louis, MO) was added to the medium. A fusion protein between a maltose binding protein (MBP) and protein GRAB was produced upon induction with IPTG.

The fusion protein was purified by affinity chromatography on an amylase resin. The fusion, MPB-Grab, Protein G and MSP-α chain of β galactosidase were

subjected to SDS-PAGE and stained with commassie. An identical SDS-PAGE was blotted to a nitrocellulose filter, and the filter was probed with radiolabeled α_2M. The predicted size of MBP-GRAB is 60 kD but it migrates with an apparent size of 80kDa. Both Protein G and the MBP-GRAB fusion were found to bind α_2M while MBP was unable to bind α_2M. Similarly MBP-GRAB, protein G, and MBP were applied in slots to a nitrocellulose membrane and probed with α_2M and it could be concluded that MBP-GRAB bound α_2M while MBP did not. MBP-GRAB, but not MBP, was found to compete for the binding of radiolabeled α_2M to KTL3 bacteria (Fig 4). Thus both protein GRAB and protein G can inhibit the binding of α_2M to KTL3 bacteria indicating that the two proteins interact with the same epitope in α_2M. A peptide covering the extreme N-terminus of the mature protein GRAB (amino acids 34-56 Fig. 2B SEQ ID No 1) was synthesized and was able to compete for the binding of α_2M to KTL3 bacteria while an overlapping peptide (amino acids 49-68 in Fig 2B) did not affect the binding (Fig 4). Thus we conclude that the extreme N-terminus of protein GRAB is responsible for binding of α_2M.

Example 4

Generation of a mutant devoid of protein GRAB on its surface - A fragment of *grab* lacking the part encoding the putative cell wall attachment region was generated by PCR from the KTL3 strain using primers 2 and 4. The fragment was cut with XhoI and HindIII which exclusively cut within primers 3 and 4 respectively and cloned into the corresponding site of streptococcal suicide plasmid pFW13 to generate FW-*grab*. This generated a 468 bp internal fragment (nt 113-580 in Fig 2B) of *grab* lacking the part encoding the cell wall attachment (Fig 5). The plasmid was electroporated into E.coli, plasmid purified and 2μg of pFW-*grab* was electroporated into KTL3 bacteria for homologous recombination (Fig 5) and several kanamycin resistant transformants were obtained. Using this cloning strategy the mutant should be devoid of surface bound protein GRAB and instead secrete a truncated form (amino acids 34-174 in Fig 2B). One transformant called MR4 was selected and its ability to bind radiolabeled α_2M was completely abolished (Fig 5).

When the supernatants from an overnight culture of MR4 and KTL3 were

precipitated with TCA, subjected to SDS-PAGE, blotted to nitrocellulose, and probed with radiolabeled α_2M it was found that the MR4 strain secreted an α_2M binding protein of 32 kDa which was not found in the KTL3 medium. The predicted size of the mature protein GRAB is 14.9 kDa, but apparently it migrates much slower in SDS-PAGE which is in concordance with the observation that the MBP-GRAB fusion also migrates slower than predicted. MR4 and KTL3 bacteria had similar growth characteristics in THY medium and the mutant survived as well as the wild type in fresh human blood (data not shown).

Example 5

Hybridization protocol is carried out as follow. Streptococci were grown in Todd-Hewitt broth with 0.2% yeast extract (THY) in 5% CO_2 at 37°C. Genomic DNA was prepared from *S.pyogenes*. 20 μg of DNA was cleaved by *EcoRI* and subjected to agarose gel electrophoresis and capillary blotting onto Hybond-N filters (Amersham, Amersham, UK). A probe was generated by PCR using *Taq* polymerase and synthetic oligonucleotides with sequences GACTCACCTATCGAACAGCCTCG and AGCTTCTTCTGATTGTAAAGCG, hybridising to *grab*. The PCR product was purified on a MicroSpin™ S-200 HR column and was radiolabeled with [α-32P]dATP using bacteriophage T4 polymerase. Membrane was prehybridized in a solution of 6xSSC, 0.5% SDS, 5xDenharts solution, and 100μg/ml salmon sperm DNA at 50°C for two hours. Probe was boiled for five minutes and added to a solution of 6xSSC, 0.5% SDS and 5xDenharts solution and incubated for 14 hours at 65°C. This was followed by washing at room temperature in 2xSSC+0.5% SDS for five minutes and 2xSSC+0.1% SDS for 15 minutes. Further washes were performed in 0.1xSSC+0.5% SDS at 37°C for one hour and in 0.1xSSC+0.1% SDS at 53°C for 30 minutes. Filter was air dried followed by exposure on BAS-III imaging plate and scanning with Bio-Imaging Analyser BAS-2000.

Example 6

α_2M is active and protects the M protein from tryptic digestion when bound to

protein GRAB - 10^9 KTL3 or MR4 cells were incubated for 40 minutes with 20 μg α_2M and carefully washed with PBS. Bound α_2M was eluted using 0.1 glycine pH 2 and subjected to SDS-PAGE. In parallel, 0.3μg of trypsin was added to the α_2M treated bacteria and the trypsin was allowed to react with surface bound α_2M for 5 minutes. Free trypsin (not in complex with α_2M) was blocked by adding a fourfold molar excess of SBTI. Cells were pelleted by centrifugation and the resulting pellet was washed once in 1 ml of PBS and resuspended in 150 μl PBS supplemented with 40μg of chloramphenicol/ml. The remaining activity of trypsin in the supernatant and the resuspended pellet was determined using the chromogenic substrate Nα-bensoyl-L-arginine p-nitroanilide (L-BAPNA) at a concentration of 0.25 mg/ml by measuring OD_{405} after three hours. The obtained value for MR4 was subtracted from that of KTL3 and compared to a standard, where the same assay was run in parallel using purified α_2M of known concentration (0.5μg). For protection assays bacteria were preincubated with α_2M as above, treated with 0.1μg of trypsin in PBS with chloramphenicol as above for 60 minutes at 37°C. Bacteria were diluted 10 times in PBSAT supplemented with 10 mM benzamidine and chloramphenicol as above and $2x10^6$ bacteria were subjected to a binding assay using radiolabeled fibrinogen.

It was found that roughly 0.5μg of α_2M was bound to 10^9 KTL3 bacteria while no band was seen in the eluate from MR4. In parallel, the amount of active α_2M bound was estimated by calculating the amounts of α_2M trapped trypsin. This L-BAPNA assay showed that 10^9 KTL3 bacteria bound 0.27+/-0.03 μg of α_2M, which correlates well with what could be eluted from the bacteria.

The complex between trypsin and α_2M was released from the KTL3 surface since all trypsin activity was found in the supernatant. To determine if this was due to release of th trypsin-α_2M complex from protein GRAB or tryptic degradation of protein GRAB, KTL3 cells were treated with trypsin and SBTI, washed, incubated with α_2M, and bound α_2M was eluted. No α_2M was bound to the trypsin treated cells indicating that protein GRAB was digested by trypsin. Thus it was concluded that α_2M bound to the surface of KTL3 is active and that protein GRAB is sensitive to trypsin treatment.

A characteristic of *S. pyogenes* M-proteins are their susceptibility to trypsin

degradation. This led us to investigate whether preincubation of KTL3 bacteria with α_2M could protect the M protein, and thus fibrinogen binding, from proteolytic degradation by trypsin. It was found that the fibrinogen binding of KTL3 could be preserved by α_2M pretreatment, while the fibrinogen binding of MR4 was unaffected by α_2M pretreatment (Fig 6).

Example 7

SCP is trapped by α_2M in solution or α_2M bound to S. pyogenes -

Radiolabeled SCP was activated in activation buffer (1 mM EDTA, and 10 mM DTT in 0.1 M NaAc-HAc, pH 5.0) for 30 minutes at 40°C. Activated SCP (4μl) was mixed with either 4 μg α_2M or 2 μl of plasma in 20 μl PBS, allowed to react for 15 minutes at 37°C, and subjected to SDS-PAGE using non-reducing conditions followed by autoradiography. Alternatively $2x10^9$ bacteria were pretreated with 40 μg α_2M, washed, and incubated with radiolabeled and activated SCP for 15 minutes. Bacteria were pelleted by centrifugation and pellet was washed with 2 ml of PBSAT and recentrifuged. Radioactivity of the pellet was measured and bound material was released by suspension of pellet in non-reducing SDS-PAGE sample buffer. Eluate was subjected to SDS-PAGE and autoradiography.

As outlined above, radiolabeled and activated SCP was mixed with either purified α_2M or with plasma and subjected to non-reducing SDS-PAGE and autoradiography. Radiolabeled SCP and α_2M were separated on the same gel as a reference. Part of the radioactivity could be seen as a band with the apparent size of α_2M indicating that a covalent complex had been formed between SCP and α_2M. Pretreatment of KTL3 and MR4 with α_2M resulted in an increased binding of SCP to KTL3, but not MR4, bacteria (Fig 7). When bound material was eluted from these bacteria, subjected to SDS-PAGE and autoradiography (as before using radiolabeled SCP and α_2M as a reference), it was found that SCP was in complex with α_2M in the case of KTL3, but not in MR4. The supernatants were separated on the same gel, and a small proportion of the radioactivity, from the α_2M pretreated KTL3 bacteria, could be seen as band with the apparent size of α_2M (data not shown). Thus we conclude that α_2M in solution or bound to *S. pyogenes* via protein GRAB can trap,

and probably also inhibit SCP.

Example 8

Generation of protein GRAB antiserum. The part of protein GRAB encoding aa 34-188 (Fig 2B) was PCR amplified from the KTL3 strain and cloned into the pET11d vector (Pharmacia Biotech, Uppsala, Sweden). Sequencing of the plasmid insert confirmed that the cloned gene was identical to *grab* from the KTL3 strain. Resulting *Escherichia coli* (BL21, Pharmacia Biotech) transformants were grown in 2xYT to OD_{620} of 0.5 and induced using 0.5 mM IPTG. Bacteria were harvested after 3 hours by centrifugation and resuspended in 20mM Tris-HCl pH 8. Bacteria were sonicated and recentrifuged at 8000xg. The bacterial lysate was subjected to ion-exchange chromatography using a mono Q column and a FPLC system (Pharmacia Biotech). Protein GRAB could be purified to approximately 90% homogeneity.

100μg of protein GRAB, from the ion exchange chromatography, in 500 μl saline was supplemented with 330 μl complete and 170 μl incomplete Freund's adjuvans and material was used to immunize one rabbit. Rabbit was boostered after 6 weeks with 100 μg of protein GRAB in 500 μl saline supplemented 500 μl incomplete Freund's adjuvans. Blood was drawn 2 weeks after boostering and serum was prepared. Serum was used in ELISA experiments where 1 ng of protein GRAB or malose binding protein (MBP, purified from the same strain of E.coli) in 50mM carbonate buffer, pH 9.6 was absorbed to Maxisorb plates (Nunc) at 4°C overnight. Wells were blocked for 1 hour at room temperature using 200μl of PBS+0.05% Tween 20 (PBST), 1% (w/v) BSA (Sigma) and incubated with varying amounts of protein GRAB antiserum or preimmune serum in the same buffer for 2 hours. This was followed by five rounds of washing with PBST and incubation with a peroxidase labelled goat antirabbit antibody (1:3000 in PBST+1 % BSA) for 1 hour at room temperature. After another round of washing 200μl of developing solution (1mg ABTS and 6 mg hydrogen peroxide/ml of Na-citrate pH 4.5) was added to each well and OD_{405} was determined after 20 minutes of incubation at room temperature. Values over 0.3 were regarded as positives. Titer of the preimmune serum was <1:100 and titer of the immune serum was >1:128 000 for protein GRAB and 1:4000

for MBP.

Similarly KTL3 or MR4 bacteria were heat killed at 65°C and 10^8 bacteria were absorbed (as above) to each well. ELISA was performed as above with the exception that protein A (1:5000) was used instead of the secondary antibody. Titer of the preimmune serum was 1:200 for KTL3 and 1:100 for MR4. Titer of the immune serum was 1:4000 for KTL3 and <1:1000 for MR4,

The antiserum was further used for western blotting of a membrane prepared as in Example 4. The filter was blocked for 30 minutes at 37°C using PBST with 5% skimmed milk. Immune or preimmune serum was diluted 1:1000 in the blocking buffer and the filter was incubated for 30 minutes at 37°C. The filter was subsequently washed three times for 10 minutes at 37°C using PBST with 0.5M NaCl. Incubation with a peroxidise labelled goat anti rabbit antibody (1:3000 in blocking buffer) was performed for 30 minutes at 37°C, followed by washing as above. Membranes were incubated with freshly made substrate consisting of 500 μl of 44.4 mM p-Coumaric acid, 100μl 250 mM Luminol (5-amino-2-3-dihydro-1, 4-phtalazinedione), and 6.1 μl of 30% H_2O_2 dissolved in 20ml Tris-HCl pH 8. Membranes were incubated for one minuted at room temperature, dried and put in a plastic bag for exposure on XAR film (Kodak). The preimmune serum showed no reactivity, whereas the immune serum specifically reacted with a band of the same size as the α_2M- binding protein in Example 4.

Example 9

The purpose of this study was to determine whether sheep immunised with various GRAB peptides, produced IgG antibodies which have the ability to bind to the corresponding peptide or native GRAB protein and whether the IgG antibodies could be titrated out. 1mg of the relevant peptide in 1.3ml saline and 3.25ml of Freund's complete/incomplete adjuvant was used for each immunisation.

Boosters were given at 3 weeks intervals with 0.5mg peptide in 1.3ml saline and 3.25ml adjuvant. The immunisation mixture was injected at 6 subcutaneous sites for each sheep. The peptides used were as follows:

Spy-PG-EKL24 (37-61)	EKLALRNEER AIDELKKQAI EDKE C*-COOH
Spy-PG-EKL 18 (37-55)	EKLALRNEER AIDELKKQ C* -COOH
Spy-PG-EER 17 (44-61)	EERAIDELKK QAIEDKE C* -CCOH
Spy-PG-DSP 18 (13-31)	DSPIEQPRII PNGGTLTN C* -COOH
Spy-PG-KKT 19 (141-160)	KKTKDTKPVV KKEERQNVN C* -COOH

C* cysteine insert for attachment to a hetero-bifunctional linker. Peptides are linked to KLH. keyhole limpet hemocyanin.

Titration and Inhibition ELISA protocol for analysis of anti-GRAB peptide anti-sera.

GRAB protein was coated onto microtitre plates (100µl/well) at a concentration of 1µg/ml, in 0.05M carbonate-bicarbonate buffer pH 9.6. The plates were incubated for 1 hour at 37°C. The plates were then washed x5 with PBS-T (250µl/well) and blocked with 1% BSA/PBS-T (100µl/well) for 1 hour at 37°C.

After washing the plates x5 with PBS-T, pre and post immune sera from sheep immunised with peptide conjugate vaccine candidates including FCA/Spy-PG-EKL24-KLH, FCA/Spy-PG-EKL 18-KLH, FCA/Spy-PG-EER 17 -KLH, FCA/Spy-PG-DSP18-KLH and FCA/Spy-PG-KKT19-KLH were diluted from 1/100 to 1/1,000,000 in PBS-T. The sera were then incubated on the GRAB coated plates (100µl sera/well) for 1 hour, at 37°C. The plates were washed x5 with PBS-T and incubated with donkey anti-sheep IgG/peroxidase conjugate (1/1000 in PBS-T) for 1 hour at 37°C. The plates were then incubated with 0.1mg/ml TMB substrate (100µl/well) for 10 minutes and then the reaction was stopped with 2M H_2SO_4 (50µl/well). Absorbances were read at 450nm.

For an inhibition ELISA, post immune sera from sheep immunised with the peptide conjugates mentioned above, were pre-incubated at 37°C, for 1 hour, at a dilution of 1/10,000, with the corresponding free peptide at concentrations ranging from 0 to 10 µg/ml. For controls, post immune sera (1/10,000) were incubated with Spy-PH-QKQ19 (10µg/ml). This peptide has the sequence QKQQQLETEKQISEASRKS C* -COOH. The serum peptide mixtures were then assayed on GRAB coated plates, as previously indicated.

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In a following inhibition ELISA post immune sheep sera were incubated with the corresponding raw peptide or the Spy-PH-QKQ19 control peptide, at a concentration of 100 μg/ml.

At dilutions of 1/100 and 1/1000, absorbances of both pre and post immune sera were generally similar. Large differences between pre and post immune serum absorbances, were often observed at dilutions of 1/10,000 and 1/100,000. Figure 8 shows the results of the assay of sheep anti- DSP18-peptide sera on a GRAB coated plate.

Inhibition ELISA's confirmed that in all cases GRAB binding antibodies in the post immune sera, could be prevented from binding whole GRAB protein, by the addition of the corresponding raw peptide. The results of the ELISA where 100μ/ml of raw peptide was added to the sera is shown in Table 1 below. When 100μ/ml of raw peptide was added to the sera, % inhibition levels generally exceeded 80%, demonstrating that high proportions of the IgG antibodies present in the serum samples were peptide specific.

Table 1. Inhibition of anti GRAB peptide antibody binding to GRAB protein, by raw peptide (100µg/ml)

		Mean absorbance 450nm			% Inhibition	
Sheep No.	Peptide Immunogen	Unabsorbed	100µg/ml relevant peptide	100µg/ml control peptide	100µg/ml relevant peptide	100µg/ml control peptide
2529-3	Spy-PG-EKL24	1.719	0.321	1.694	81.3	1.5
2530-3	Spy-PG-EKL24	1.924	0.274	1.730	85.8	10.1
2531-3	Spy-PG-EKL18	1.515	0.207	1.598	86.3	0
2532-3	Spy-PG-EKL18	1.508	0.267	1.538	82.3	0
2533-3	Spy-PG-EER17	1.693	0.297	1.454	82.5	14.1
2631-3	Spy-PG-EER17	1.656	0.259	1.590	84.4	4.0
2535-3	Spy-PG-DSP18	1.591	0.201	1.665	87.4	
2596-3	Spy-PG-DSP18	1.752	0.492	1.676	71.9	4.3
2597-3	Spy-PG-KKT19	0.763	0.177	0.780	76.8	0
2598-3	Spy-PG-KKT19	1.469	0.210	1.689	85.7	0

NB. The control peptide used was Spy-PH-QKQ19

Titration ELISA protocol.

Peptide was coated onto microtitre plates (100µl/well) at a concentration of 5µg/ml, in 0.05M carbonate-bicarbonate buffer pH 9.6. The plates were incubated for 1 hour at 37°C. The plates were then washed x5 with PBS-T (250µl/well) and blocked with 1% BSA/PBS-T (100µl/well) for 1 hour at 37°C.

After washing the plates x3 with PBS-T, pre and post immune sera from sheep immunised with peptide conjugate vaccine candidates including FCA/Spy-PG-EKL24-KLH, FCA/Spy-PG-EKL18-KLH, FCA/Spy-PG-EER 17-KLH, FCA/Spy-PG-DSP 18-KLH and FCA/Spy-PG-KKT19-KLH were diluted from 1/100 to 1/1,000,000 in PBS-T. The sera were then incubated on plates coated with the corresponding peptide (100µl sera/well) for 1 hour, at 37°C. The plates were washed x3 with PBS-T and incubated with donkey anti-sheep IgG/peroxide conjugate (1/1000 in PBS-T) for 1 hour at 37°C. After washing x5 with PBS-T, the plates were incubated with 0.1mg/ml TMB substrate (100µl/well) for 10 minutes and then the reaction was stopped with 2M H_2SO_4 (50µl/well). Absorbances were read at 450nm.

The results obtained from the experiment described above for Spy-PG-EKL 24 are presented in figure 9 and have been summarised for all peptides in table 2 below.

Table 2. Sheep anti-GRAB peptide antibody titres as determined by ELISA

Sheep No.	Pre/post	Peptide immunogen	Assay asborsbance cut off point	Antibody Titre
2529	pre		0.45	1 in 100
2529-3	post	Spy-PG-EKL24	0.45	1 in 1,000,000
2530	pre		0.45	1 in 100
2530-3	post	Spy-PG-EKL24	0.45	1 in 1,000,000
2531	pre		0.45	1 in 100
2531-3	post	Spy-PG-EKL18	0.45	1 in 100
2532	pre		0.45	1 in 1000
2532-3	post	Spy-PG-EKL18	0.45	1 in 1,000,000
2533	pre		0.45	1 in 1000
2533-3	post	Spy-PG-EER17	0.45	1 in 1,000,000
2631	pre		0.45	1 in 100
2631-3	post	Spy-PG-EER17	0.45	1 in 100,000
2535	pre		0.30	1 in 1000
2535-3	post	Spy-PG-DSP18	0.30	1 in 1,000,000
2596	pre		0.30	1 in 1000
2596-3	post	Spy-PG-DSP18	0.30	1 in 1,000,000
2597	pre		0.15	1 in 1,000,000
2597-3	post	Spy-PG-KKT19	0.15	1 in 1,000,000
2598	pre		0.15	1 in 1000
2598-3	post	Spy-PG-KKT19	0.15	1 in 1,000,000

Example 10

Immunisation studies

Mice (5-6 weeks old), 10 per group, were immunised subcutaneously (sc) at the tail base with 50 µl of vaccine emulsion containing 30 µg of peptide/protein/peptide-conjugates emulsified in CFA. Control mice are given PBS in CFA. Peptides emulsified in Complete Freund's adjuvant (CFA, H37Ra, Difco Laboratories Cat# 3113-60-5) were prepared as follows:-

33 µl of peptide (10 mg/ml stock) and 517 µl of sterile PBS with 550 µl of CFA were mixed in an eppendorf. Using a 1 ml syringe with 18G needle materials was homogenized until the volume was reduced by half. Mixture may be tested by centrifuging in eppendorf for 1 min at 1000 rpm, if mixture does not separate it is OK to proceed. Alternatively, one drop of emulsion is placed on water. If emulsified, drop should remain tight and not disperse. The emulsified mixture was drawn into same syringe, tuberculin needle fixed, and air bubbles removed.

Mice were given booster injections at days 23 and 30, sc, of 30 µg and 15 µg respectively of peptide/protein/peptide conjugates dissolved in PBS.

Mice were bled at Day 14, Day 23, Day 29 and Day 38 via the tail artery and sera are prepared and stored at - 20° as follows. Murine blood was collected into eppendorf tubes (100-300 µl) via scalpel cut to the tail artery. The blood was allowed to clot either overnight at 4°C or for 1 hour at 37°C. The blood clot was picked out and discarded with sterile toothpick or pipette tip, eppendorf tube was spun at 3000 rpm for 10 minutes. The sera was removed (clear supernatant) to fresh tube. Short term storage < 1 week at 4°C, long term storage at - 20°C. ELISA was carried out as described below in Example 11.

The results are shown in Figure 10.

Opsonization was carried out using blood from the day 47 bleed. A 100 µl aliquot of stock GAS (Group A streptococcus) was cultured overnight in 5 ml of sterile Todd Hewitt Broth (THB)/1% Neopeptone at 37°C. To use log phase growth bacteria in the assay, 20 µl of the overnight GAS culture was subinoculated into 5 ml of THB/1% Neopeptone that was pre-warmed at 37°C. GAS were grown at 37°C

for 2 hours. GAS (either log phase GAS [2 hour culture] or stationary phase GAS [from the overnight culture]) were diluated in sterile saline to 10^{-5}.

Fifty µl of the 10^{-4} and 10^{-5} bacterial dulution was mixed with 50 µl of heat inactivated (60°C for 10 minutes) normal mouse serum or immune mouse serum, mixed well, and incubated at room termperature for 20 minutes.

400 µl of normal heparinised human blood (pre tested to be non-opsonic for the strain of GAS used in the assay) was added and the mixture was incubated with end-to-end rocking at 37°C for 3 hours. 50 µl of the bacterial dilution was plated out mixed in a petri-dish with 15 ml molten 2.5% blood THB agar. 50 µl of the 10^{-4} bacterial dilution was held at 4°C until it was plated out to estimate inoculum size. The plates were incubated at 37°C overnight. Mean colony count was determined by counting colonies on plates. The percentage reduction in colony-forming units (CFU) of bacteria is calculated by comparing means colony counts after incubation with mouse immune serum compared with normal mouse serum multiplied by the dilution factor.

Tests were carried out as follows:

Number of sera tested in the opsonisation assay per immunisation group:

- GRAB protein n=3
- EKL24-KLH, n=10
- DSP18-KLH, n=6
- KKT19-KLH, n=10
- PepM 88/30 n=3. Sera from mice immunised with PepM derived from 88/30 GAS was used as the positive control in the assay.

The results are shown in Figure 11. The mean is ± sem.

Example 11

Additional studies were carried out specifically to look for an antibody in human sera having specificity to a C-terminally adjacent region to the α_2M binding site of protein GRAB. Such an antibody should be able to bind to protein GRAB as this region should be available on the surface of GAS. In view if the studies described in Example 10, EKL24 was studied further in a human population. Human

studies focussed on the pre-existing immunity of an endemic human population (Thailand) highly exposed to group A streptococcal infections. Table 3 below shows that sera from Thai individuals with rhematic heart disease (RHD) and healthy individuals with no heart disease, alike, both contained antibodies to EKL24. Antibody titers were measured by ELISA as set out below.

Antigen (peptide/protein) was diluted to 5 µg/ml in carbonate-bicarbonate buffer. For example, 5 µl of peptide from a 10 mg/ml peptide stock was added to 10 ml of carbonate-biocarbonate buffer (enough for one plate). 100 µl per well was added to flat bottomed polyvinyl chloride microplates (Flow Laboratories Inc.) and incubated overnight at 4°C or 90 mins at 37°C. Antigen was flicked off the plate and the wells were blocked with 200 µl of 5% skim milk in PBS-Tween 20 overnight at 4°C or 90 mins at 37°C. Plates were washed 3 times with PBS-Tween 20. Human or mouse sera are diluted 1:100 in the first row and serially diluted 1:2 down the plate to 1:12800 in a final volume of 100 µl.

Plates with primary antibody are then incubated at 37°C for 90 minutes. Plates are washed 5 times with PBS-Tween 20. If using human sera, goat anti-human IgG/HRP (Bio-rad) or if using mouse sera, goat anti-mouse IgG (Amrad) was diluted 1:3000 in 0.5% Skim milk/PBS-Tween 20. 100 µl is added to each well and incubated at 37°C for 90 minutes. Plates are washed 5 times with PBS-Tween 20. 100 µl of OPD substrate (OPD FAST, Sigma-OPD and buffer tablets supplied with kit) was added to each well and incubated in the dark at room temperature for 30 minutes. The optical density was measured at 450 nm. For human antibodies, antigen-specific antibody concentration is calculated using standard curves of optical density versus known concentrations of human IgG for murine antibodies, a value of titre is used to measure quantity of antibody and is defined as the mean plus three standard deviations of the normal mouse sera wells.

The results are set out in Table 3 below.

Table 3

Serum Antibody Response to peptide Spy-PG-EKL24 (37-61) in Control and RHD Thais.

Number of Individuals with an antibody response:	RHD	Controls
Titre ≤400	45/62	19/35
Titre 800-1600	8/62	16/35
Titre ≥ 3200	9/62	0/35

ELISA was used to measure human serum antibodies to the peptide. The titre is defined as the mean plus three standard deviations of the blank (no antibody) wells.

Subsequently, T-cell proliferation assays were carried out.

Thirty ml of heparinised blood is split between two 50 ml Falcon tubes with conical base and diluted 1:2 (15 ml) in sterile PBS. Blood is underlayed with 10 ml of Ficoll (at room temperature).

Cells are separated by centrifugation at room temperature, 1200 rpm for 30 minutes (with brake off). PBMC layer from both tubes are removed with a sterile pipette and pooled into a single 50 ml Falcon tube, diluted to 50 ml of sterile PBS and centrifuged at 1500 prm for 10 minutes.

The supernatent is discarded and PBMC are resuspended in 5 ml sterile RPMI/10% normal human sera (NHS). NHS has been heat-inactivated for 20 minutes at 60°C added to media to 10% in RPMI and filter-sterilised prior to use in the assay.

Cells are counted and resuspended in RPMI/10%NHS with 100 µg/ml streptomycin/1000U/ml penicillin /2.5mcg/ml fungizone (CSL:catalogue # 0929501), a final concentration of 1 X 10^6 cells/ml.

Peptides/proteins were plated out onto round bottomed 96 well plates at pre-determined optimal concentrations (30 µg/well of peptide). Wells without antigen were also included. 200 µl of PBMC, at a final concentration of 2 X 10^5 cell/well,

were added to 96 well plates containing the peptide/proteins. After 4 days of culture at 37°C in 5% CO_2, 25 µl of culture supernatant can be removed from each well for cytokine analysis. After 6 days of culture 0.25 µCi ^3H methyl-thymidine was added to each well and 16 hours later incorporation of label was measured by liquid scintillation spectroscopy. Cells are harvested onto a filter mat, filters are dried then sealed in plastic bags with 12 ml of scintillant and counted in a LKB Wallac 1205 Betaplate liquid scintillation counter. The mean cpm of triplicate wells with peptide/protein was divided by the mean cpm of 6 wells without peptide to give a stimulation index (SI). A SI of 5 was used as a cut-off for significant proliferative response in adult subjects as previously described (Pruksakorn S, Currie B, Brandt E, Phornphutkul C, Hunsakunachi S, Manmontri A, Robinson JH, Kehoe MA, Galbraith A, Good MF. *Int. Immunol.* 1994; 6: 1235-44).

The assay demonstrated that PBMC from RHD patient in the population recognised EKL24. Results are set out in Table 4 below.

Table 4

Proliferative response of PMBC From Control and RHD Thais to peptide Spy-PG-EKL24 (37-61).

Number of Individuals with a	RHD	Controls
Stimulation Index >5	4/62	0/35

The sequences mentioned herein are set out in the sequence listing below and can be summarised as follows:

SEQ ID No. 1 is the amino acid sequence of positions 34-56 inclusive of strain SF370 as set out in Figure 2B.

SEQ ID No. 2 is the amino acid sequence of positions 34-91 inclusive of strain SF370 as set out in Figure 2B.

SEQ ID No. 3 is the amino acid sequence of positions 92-119 inclusive of strain SF370 as set out in Figure 2B and is one of the repeat sequences of the protein.

SEQ ID No. 4 is the amino acid sequence of positions 34-217 inclusive of strain SF370 as set out in Figure 2B and is the full length mature protein i.e. without the signal sequence.

SEQ ID No. 5 is the amino acid sequence of positions 34-174 inclusive of strain SF370 as set out in Figure 2B. This truncated form of the protein is missing the trans-membrane and wall anchor regions.

SEQ ID No. 6 is the amino acid sequence of positions 34-193 inclusive of strain SF370 as set out in Figure 2B, and does not include the membrane spanning region of the protein.

SEQ ID No. 7 is the amino acid sequence of the full length protein of strain SF370 as set out in Figure 2B including signal sequence.

SEQ ID Nos. 8-11 are partial amino acid sequences for protein GRAB derived from strains KTL9, AP1, AP49 and KTL3 respectively.

SEQ ID Nos. 12-16 are DNA sequences encoding the amino acid sequences of SEQ ID Nos. 7-11 respectively.

SEQ ID Nos. 17-21 are primers derived from SEQ ID No. 12.

SEQ ID No. 22 is the amino acid sequence for the peptide DSP18

SEQ ID No. 23 is the amino acid sequence for the peptide EKL 24

SEQ ID No. 24 is the amino acid sequence for the peptide EKL 18

SEQ ID No.25 is the amino acid sequence for the peptide EER 17

SEQ ID No. 26 is the amino acid sequence for the peptide KKT19